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Research Article

Shrinking pupal cocoons of *Rhyacophila lezeyi* (Trichoptera, Rhyacophilidae) in a highly acidic stream during the summer season*

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Abstract

Shrinking pupal cocoons of *Rhyacophila lezeyi* were often found during summer in Shibukuro Stream, a highly acidic mountain stream in northern Japan (pH = 2.82 on average). We performed both field surveys and laboratory rearing experiments to clarify the mechanisms of *R. lezeyi* cocoon shrinkage. The *R. lezeyi* cocoon shrinkage proportion increased in years with high stream water temperatures and was related to water temperatures before and after pupation at the study site. Approximately 90% of the prepupae and pupae inside the shrinking cocoons died during the rearing experiment, implying that cocoon shrinkage caused by high water temperature strongly influenced *R. lezeyi* pupal survival. Laboratory experiments showed that *R. lezeyi*'s pupal cocoon membranes were semi-permeable and that the cocoon fluids were always hyperosmotic, indicating that water molecules can continuously enter the cocoon fluids from the stream water until the turgor of the cocoon wall is reached. However, the shrinking cocoons showed lower fluid volume and higher osmolarity than the normal turgescent cocoons. The reduction of osmotic gradient across the membrane during decreased stream flow due to less precipitation and/or the damage to the cocoon membrane and pupal body from high and fluctuating water temperatures and low pH are possible mechanisms for *R. lezeyi* pupal cocoon shrinkage.

Key Words

cocoon shrinkage, membrane semi-permeability, osmolarity, pH, water temperature

Introduction

The eastern and northern parts of Japan have many acidic mountain streams because of the high volcanic activity in the region (Goto 1982). *Rhyacophila lezeyi*, Navás, 1933, often inhabits such extreme environments (Tsutsumi and Yokoyama 2020). One of these is Shibukuro Stream, where stream water is a mixture of a nearby hot-acidic spring water with high temperature (97°C) and low pH (pH 1.1) and streams diluting those conditions. In the middle reaches of Shibukuro Stream, *R. lezeyi* larvae and pupae occur in high densities, predominating the faunal communities' biomass (Aoya 2018). *Rhyacophila lezeyi* is distributed in the south of Honshu Island and is one of the most common species of the genus *Rhyacophila* in Japan, except the northern region (Hattori 2005). The larvae and pupae of *R. lezeyi* are confined to cool stenothermic running waters. The larvae feed mainly on nemourid stonefly nymphs in Shibukuro Stream, often exerting strong predation pressure on the lower trophic levels (Aoya 2018). *R. lezeyi* has an asynchronous univoltine life cycle and its larvae pupate and emerge

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continuously from May to October, peaking in June, in the stream (Aoya and Tanida 2023). The final instar larva of *R. lezeyi* normally constructs a turgescent pupal cocoon (Fig. 1A) inside the pupal case and the pharate adult breaks out from the cocoon for emergence (Aoya 2020; Aoya 2022). However, shrinking cocoons (Fig. 1B) are frequently found in Shibukuro Stream in summer and the cocoon shrinkage mechanisms have yet to be elucidated.

Figure 1. *Rhyacophila lezeyi* pupae inside cocoons collected in Shibukuro Stream. 1A: Normal (turgescent) cocoon (29 Aug 2018). 1B: Shrinking cocoon (19 Aug 2019). The scale is 5 mm.

We performed both field surveys and laboratory experiments to identify plausible mechanisms that cause the *R. lezeyi* summer cocoon shrinkage in Shibukuro Stream. We focused specifically on the osmotic gradient of the *R. lezeyi* pupal cocoon membrane between stream water and cocoon fluids because osmosis can supply cocoon fluids with oxygenated stream water across a semi-permeable cocoon membrane (Wichard 1991; Wichard et al. 1997), as has been reported for *Rhyacophila fasciata* Hagen, 1859 (Wichard et al. 1993). We hypothesise that high solute concentrations in stream water during summer reduce the osmotic gradient across the cocoon membrane, thereby causing the cocoon shrinkage and prepupal and pupal mortality of *R. lezeyi*.

Methods

Field surveys

We conducted a field survey at Shibukuro Stream, a tributary of Tamagawa River in Omonogawa River System, northern Japan. The study site (39.9388°N, 140.7059°E, elevation: 480 m, base-flow discharge = 1.4 m^3 s^{-1}) is located 4.5 km downstream from the hot spring water inputs from Tamagawa hot spring area (Aoya and Tanida 2023). Using a data logger (TidbiT v.2, Onset Computer Corporation, Bourne, MA, USA), we measured the water temperature at hourly intervals during 2018–2021. During our field survey visits to the site, one to five times per month from April to November, we measured the pH using a glass electrode meter (HI98108N, Hanna Instruments, Woonsocket, RI, USA). We also measured electrical conductivity (EC) using a compact EC meter (LAQUAtwin-EC-33B, Horiba, Kyoto, Japan) once or twice a month from July 2021 to June 2022, except the winter period (December to March). Additionally, we collected daily meteorological data (i.e. air temperature, sunlight hours and precipitation) from a nearby meteorological station (the Hachimantai Observatory of the Japan Meteorological Agency).

We handpicked *Rhyacophila lezeyi*'s pupal cases containing the closed prepupal and pupal cocoons at the study site from July to September 2018, 2019 and 2021. We transported the collected samples to the laboratory alive, carefully removed sand and gravel around the cocoons and picked the cocoons within three to six hours of sampling.

Laboratory procedures

In the laboratory, brownish cocoons were used for further analyses; no immature cocoons with thin membranes were examined in the present study. We checked each *R. lezeyi*'s pupal cocoon for pupal life (alive or dead) and morphological conditions (turgescent or shrinking). We reared all the living individuals in a dark refrigerator, in which the temperature was maintained at 1–6°C below the Shibukuro Stream water temperature. We also recorded the metamorphosis dates (pupation, breaking out from the cocoon and emergence) of each individual during rearing and prepupal and pupal mortality after rearing.

Cocoon membrane semi-permeability

We examined the *R. lezeyi* cocoon membranes' semi-permeability. First, we placed living immature pupae with undamaged turgescent cocoons in stream water and kept them overnight in a refrigerator at about 16°C. Second, we used distilled water (DW) to perform stepwise dilution in about one hour to finally transfer the pupae into the DW. Third, we transferred the cocoons to a saturated sodium chloride (NaCl) solution for 10 minutes and then directly returned them to the DW. We observed the changes in cocoon morphology in both the DW and saturated NaCl solutions.

Cocoon fluid analyses

We analysed the chemical composition of fluids inside the *R. lezeyi* cocoons collected at the study site on 20 July 2021. We divided the 49 cocoons containing living individuals into four types: prepupae with turgescent cocoons (n = 18), immature pupae with turgescent cocoons (n = 14), mature pupae with turgescent cocoons (n = 7) and immature pupae with shrinking cocoons (n = 10).

We pierced each cocoon membrane and, with a gentle press of the membrane, dripped the cocoon fluids on to a plastic dish. To obtain the average mass of fluids per cocoon for each cocoon type, we weighed each fluid sample. We equated 1 mg to 1 µl. Then we diluted the fluid samples, centrifuged them for 10 min at 3000 rpm and

filtered them through a 0.45 µm cellulose acetate filter (Advantec Toyo Kaisha, Ltd., Tokyo, Japan).

We measured the inorganic ion concentrations in the stream water and cocoon fluids as follows: using a continuous flow auto analyser (QuAAtro2-HR, BL TEC K.K., Osaka, Japan), we measured the NH_4^+ concentration, we quantified the other major cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) using a microwave plasma atomic emission spectrometer (4210 MP-AES, Agilent, Santa Clara, CA, USA) and we measured all the anion species using ion chromatography (DIONEX ICS-2100, Thermo Fisher Scientific, Waltham, MA, USA). We determined the bicarbonate (HCO_3^-) concentration from ion balance through the difference between the total cation equivalent and total anion equivalent. We also determined the osmolarity (mOsm l^{-1}) of the cocoon fluids and stream water from the molar concentration of each ion.

Results

Water temperature, pH and EC of Shibukuro Stream

The monthly average values (mean ± SD) for stream water temperature, pH and EC at the study site were 11.8 ± 5.0°C, 2.82 ± 0.24 and 1,572 ± 661 µS cm^{-1}, respectively. The temperature and EC values were about 4°C higher and about 20–40 times higher, respectively, than those in nearby mountain streams receiving no hot spring water. We compared annual variations in temperature and pH of the stream from July to September. Both the daily water temperature and its daily range were significantly increased and the pH was significantly decreased in 2019 and 2021 compared to those in 2018 (Table 1). Precipitation also decreased in 2019 and 2021 (Table 1). However, the air temperature and sunlight hours did not differ between each of the three years. Therefore, we attribute the observed water temperature increase and pH decrease to the decreased stream flow associated with less precipitation in 2019 and 2021 relative to 2018 (Table 1). This is because low-flow conditions may increase the relative contribution of the Tamagawa hot spring water (discharge = about 0.15 m^3 s^{-1}) to the stream flow, even though the reliable estimates for the proportion of hot spring water to river flow are lacking.

Rhyacophila lezeyi cocoon shrinkage and pupal mortality

In the summers of 2019 and 2021 with high water temperatures, *Rhyacophila lezeyi* cocoon shrinkage occurred frequently and they experienced high pupal mortality in the study stream. The average proportion of shrinking cocoons in the collected sample year was approximately 4–6 times higher in 2019 (54.1%, n = 74) and 2021 (31.5%, n = 197) than in 2018 (8.9%, n = 124). We also found a significant positive correlation between the mean water temperature for 20 days before collection and the proportion of shrinking cocoons (Fig. 2), suggesting that the factors associated with water temperature increases induced *R. lezeyi* cocoon shrinkage. Moreover, a significant positive correlation was evident between the shrinking cocoon proportion and the prepupal and pupal mortality ($r = 0.825$, $p < 0.001$) in the stream.

The laboratory rearing experiment also confirmed the extremely high mortality of prepupae and pupae in shrinking

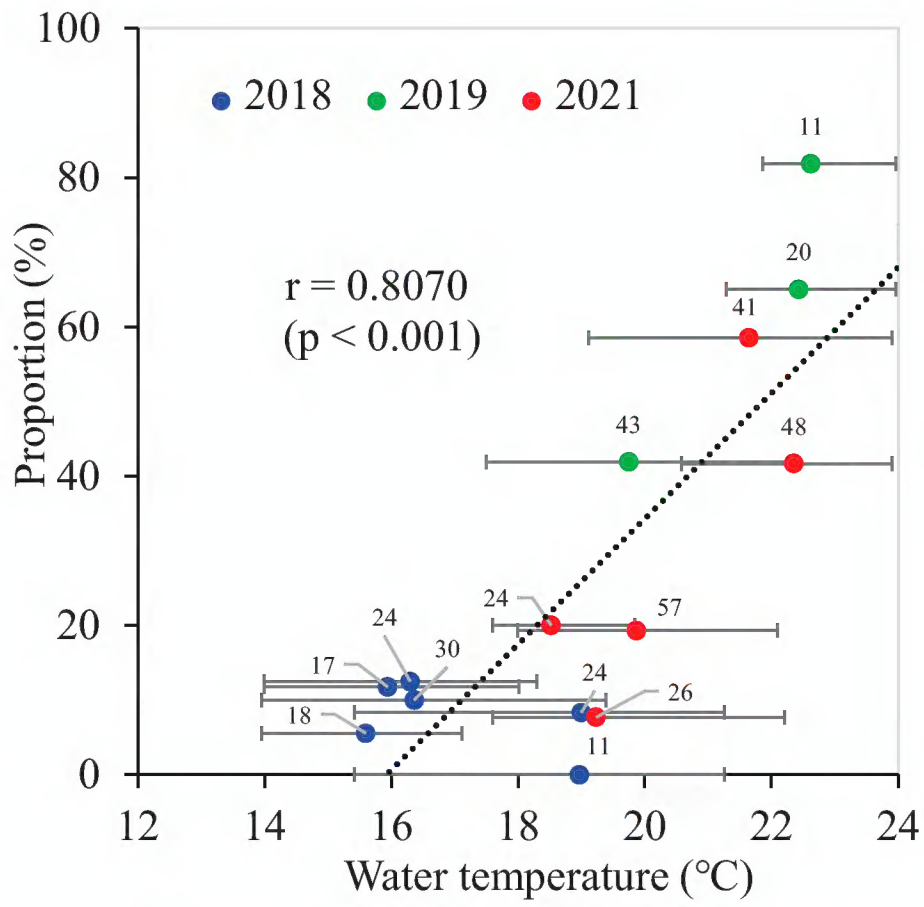

Figure 2. The relationship between the mean stream water temperatures for 20 days prior to collection days and the proportion of shrinking *Rhyacophila lezeyi* pupal cocoons from July to September (2018, 2019 and 2021). The horizontal bars show the range of the daily mean stream temperatures. The numbers above the symbols show the total number of individuals collected.

Table 1. Stream water temperature, pH and electrical conductivity (EC) at the study site of Shibukuro Stream and precipitation at a nearby weather station from July to September (2018, 2019 and 2021).

	2018	2019	2021	Significance test
Daily stream water temperature (°C)	17.6^b ± 2.02	**20.1^a** ± 1.88	**20.0^a** ± 1.78	$p < 0.001^{AN}$
Diurnal range of stream water temperature (°C)	2.26^b ± 1.02	**2.86^a** ± 1.07	**2.77^a** ± 1.09	$p < 0.001^{AN}$
pH	2.93^b ± 0.16	**2.68^a** ± 0.16	**2.68^a** ± 0.09	$p < 0.01^{KW}$
Electrical conductivity (EC) (µS cm^{-1})	—	—	2191 ± 357	
Daily precipitation (mm)*	8.02^c ± 17.3	**2.88^b** ± 6.83	**3.96^a** ± 8.22	$p < 0.01^{KW}$
				$a \times b$: $p < 0.05^{WR}$

*Hachimantai Meteorological Station; Numerals before ± are the means and numerals after ± are the standard deviations; AN: one-way ANOVA; KW: Kruskal-Wallis test; WR: Wilcoxon rank sum test.

cocoons (> 90%), although the mortality of those in turgescent cocoons was relatively low (< 10%, Table 2). Furthermore, the prepupal and pupal mortality rates were significantly higher in 2019 (i.e. a high-temperature year) than in 2018, indicating that the cocoon shrinkage caused by high water temperature strongly influenced *R. lezeyi* survival.

Cocoon membrane semi-permeability and fluid osmolarity

The experiment to confirm the cocoon membrane semi-permeability revealed that the *R. lezeyi* pupal cocoons lost fluid immediately after the transfer to the saturated NaCl solution, resulting in significant cocoon shrinkage (Fig. 3). Once we returned the shrinking cocoons to DW, they slowly regained their initial volume and turgidity and we observed no life condition damage (Fig. 3).

Under natural conditions, the mean volume of prepupal cocoon fluids (12 µl) increased to 16 µl for immature pupae and 18 µl for mature pupae. However, the immature pupae inside shrinking cocoons had less fluid volume (10 µl) than normal. Chemical analyses revealed that the cocoon fluids' ion concentrations were generally higher than those of stream water and that half the ion species (Na^+, NH_4^+, NO_3^-, HPO_4^{2-} and HCO_3^-) were only present in the cocoon fluids, indicating an internal source of such chemical substances for the fluids (Fig. 4). The normal prepupal cocoons had the highest osmolarity (91 mOsm l^{-1}), followed by normal immature pupal cocoons (54 mOsm l^{-1}), normal mature pupal cocoons (44 mOsm l^{-1}) and stream water (27 mOsm l^{-1}; Fig. 4). Shrinking cocoon fluids had higher osmolarity than the normal cocoons of immature and mature pupae (Fig. 4).

Discussion

In the highly acidic Shibukuro Stream, the increase of *Rhyacophila lezeyi* shrinking cocoons was associated with higher and fluctuating water temperatures and a lower pH (Table 1). Such changes in the physicochemical environment are probably related to increased hot spring water contributions resulting from the decreased stream flow due to less precipitation. In fact, the mean summer precipitation (July-September) was the lowest and the third lowest for 2019 and 2021, respectively, during the 1979–2022 period, while the precipitation level was the 13th highest for 2018 during that period. Larvae and pupae exposed to higher water temperatures more frequently experienced collapsed cocoons (Fig. 2). Cocoon shrinkage also was correlated with severe mortality in *R. lezeyi* (Table 2). Therefore, increased water temperature may influence *R. lezeyi* cocoon morphology and pupa physiology, which may eventually suppress their development.

One possible cocoon shrinkage mechanism was at least partially due to the change in the osmotic gradient over the cocoon membrane (Fig. 5). *Rhyacophila lezeyi*'s pupal cocoon membrane was found to be semi-permeable (Fig. 3), as was *R. fasciata*'s in Fulda River in Germany

Table 2. Mortality of prepupae and pupae of *Rhyacophila lezeyi* during the laboratory rearing experiment.

		before pupation		before breaking out from the cocoon				Total	
		prepupae		pupated prepupae		pupae			
Normal cocoon (Turgescent)	2018	2.4 %	(42)	7.1 %	(42)	9.9 %	(71)	9.7 %	(113)
	2019	0.0 %	(8)	25.0 %	(8)	0.0 %	(26)	5.9 %	(34)
Shrinking cocoon (Collapsed)	2018	75.0 %	(4)	25.0 %	(4)	85.7 %	(7)	90.9 %	(11)
	2019	73.3 %	(15)	26.7 %	(15)	92.0 %	(25)	95.0 %	(40)

Numerals in parentheses: total individuals collected; Annual comparison: $p < 0.05$ (Kruskal-Wallis test).

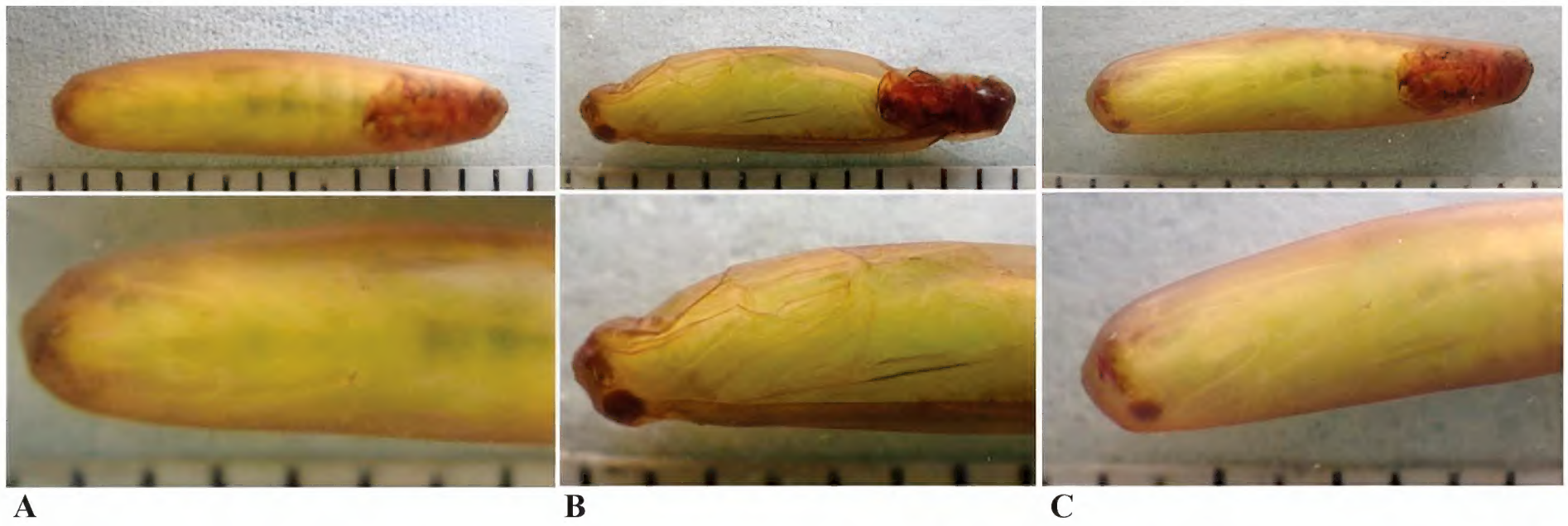

Figure 3. Photographs of *Rhyacophila lezeyi* cocoons. The scale is 1 mm. 3A: The cocoons were first kept in distilled water. 3B: Two minutes after transfer to a saturated NaCl solution. 3C: Five minutes after returning to distilled water. The water temperature was about 20°C.

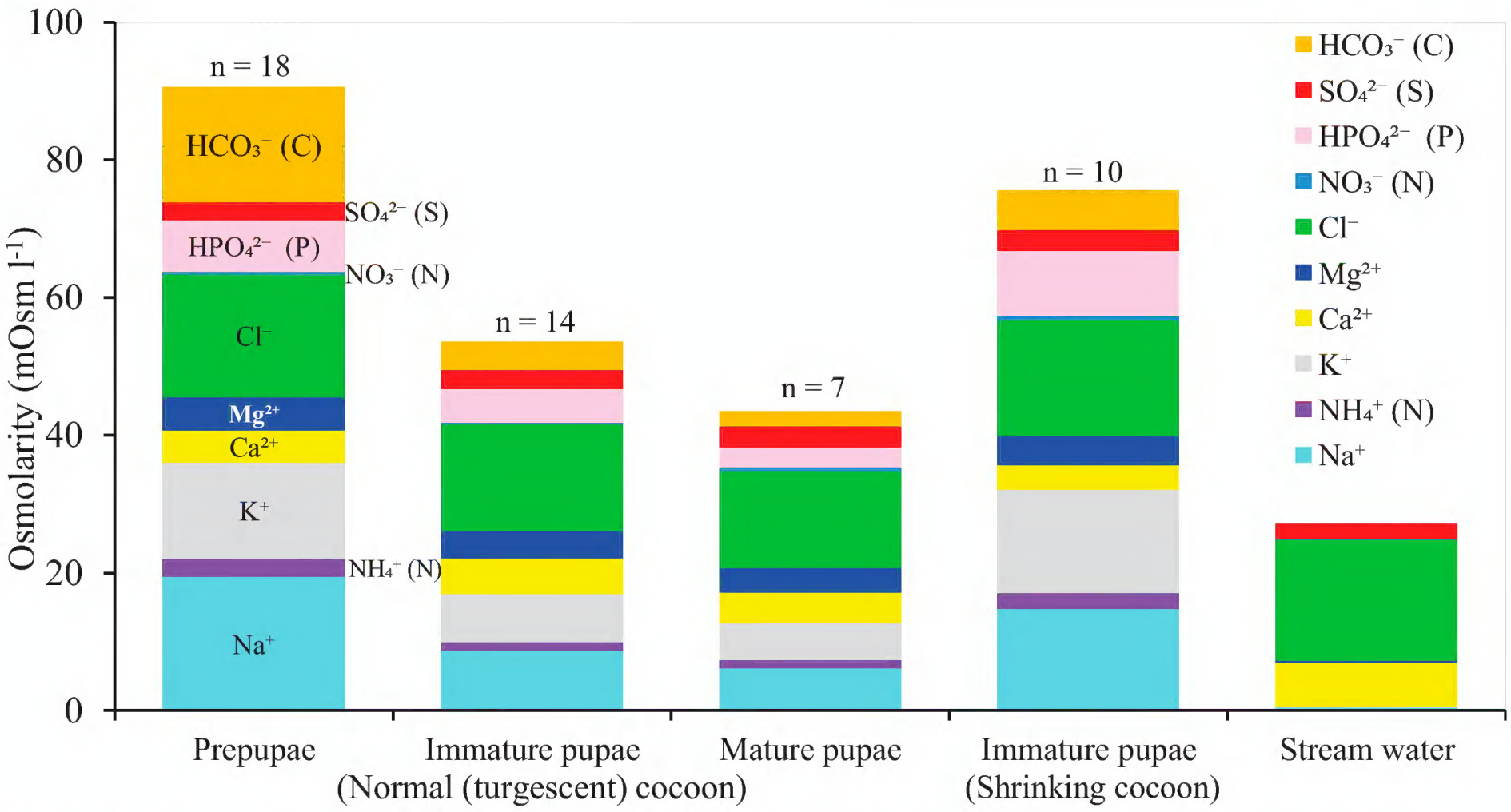

Figure 4. The osmolarity of inorganic components in the pupal cocoon fluids of *Rhyacophila lezeyi* and the surface water of Shibukuro Stream. The total number of cocoons used in the experiment (n) is shown.

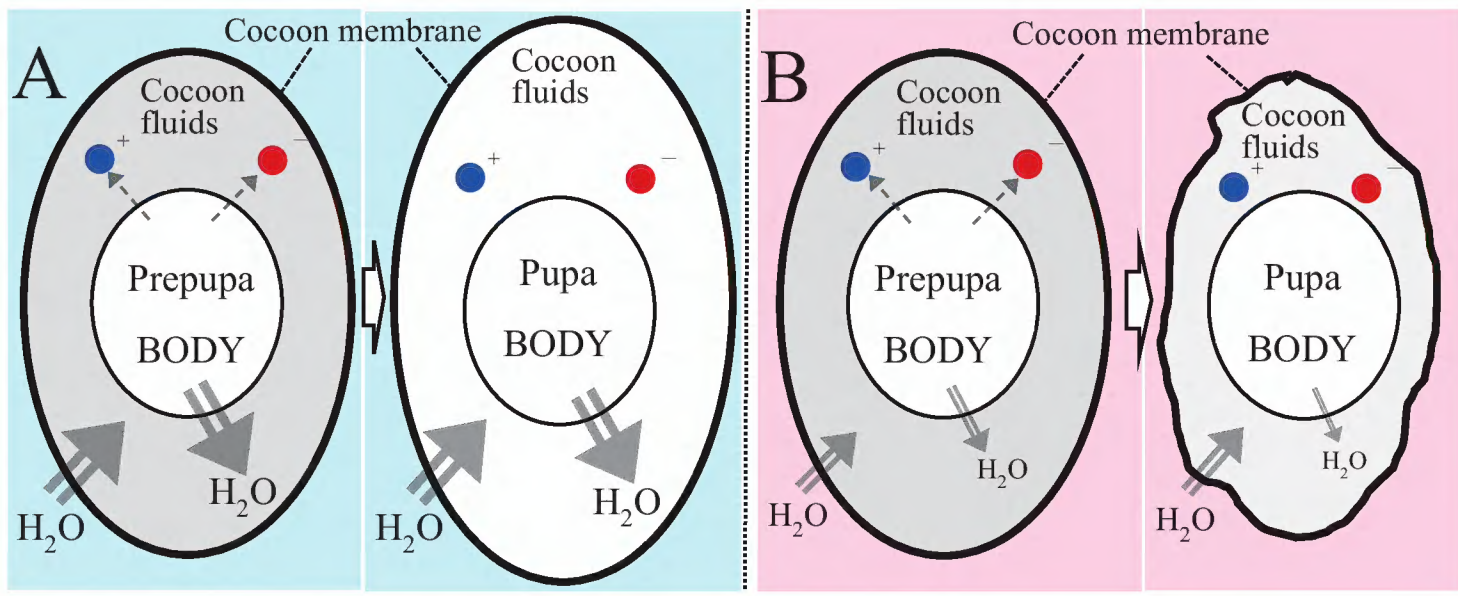

Figure 5. Plausible pathways for water and solutes in cocoon fluids, body fluids and stream water. 5A: Under normal conditions, stream water is forced into the cocoon fluids due to the large osmotic gradient across the semi-permeable membrane. As the prepupae moult, exuvial fluids rich in ions are released and the cocoon fluids are gradually diluted. The pupae might excrete excess water from their bodies into the cocoon fluids. 5B: In high-temperature water, the rate of water influx into the cocoon fluids may decrease because the reduced flow is associated with less precipitation to enrich the stream water's solute concentration. High and fluctuating water temperatures and low pH might also influence the physical and physiological properties of the cocoon and pupa, resulting in cocoon shrinkage.

(Rudolph and Wichard 1993; Wichard et al. 1993). As the observed cocoon fluids' osmolarity was always higher than that of stream water, water molecules were generally allowed to permeate from stream water into the cocoons. Wichard et al. (1993) suggested that dissolved organic matter, in addition to inorganic components in the cocoon fluids of *R. fasciata*, had an osmotic effect. Even if we consider the osmolarity of only the organic components present in the cocoon fluids of *R. lezeyi*, the water influx direction does not change. In fact, for normal turgescent cocoons, cocoon fluids' osmolarity and cocoon volume decreased and increased with pupal development, respectively (Fig. 4), implying that the cocoon fluids were diluted by stream water permeation. However, the shrinking cocoons showed lower fluid volume and higher hyperosmotic conditions than did the normal cocoons (Fig. 4). Thus, water removal from the cocoons via the membranes due to events of higher osmolarity (> 40–90 mOsm l^{-1}) of the Shibukuro Stream water might be mechanisms of pupal cocoon shrinkage and high osmolarity.

In fact, higher ion concentrations, as revealed by higher conductivity values (EC range = 2,317–2,570 µS cm^{-1}), were often observed during the summer months compared with the day of osmotic pressure measurement (20 July 2021, EC = 1,971 µS cm^{-1}). In addition, ion concentrations of stream water are likely further enriched during periods of decreased stream flow with less precipitation. Therefore, we argue that the removal of cocoon fluids via

osmoregulation is responsible for the shrinkage of the pupal cocoon under the influence of the temporary-occurring hyper-osmotic water medium in the acidic stream.

Other plausible factors for the cocoon shrinkage are high water temperature and low pH, which may cause physical and physiological stress. The increase in water temperature results in the decrease in dissolved oxygen concentration, which might affect pupal respiration (Wiggins and Wichard 1989; Wichard et al. 2002). Strong acidic conditions may weaken the cocoon threads that support morphological cocoon turgescence. The inhibition of water excretion from pupal body fluids might also be possible due to the physiological stress of high and fluctuating water temperature and a low pH (Fig. 5).

Conclusions

The present study revealed that *Rhyacophila lezeyi* cocoon shrinkage observed in the highly acidic Shibukuro Stream was associated with relatively high water temperatures and a low pH, both of which were resulted from the decreased stream flow due to low precipitation during summer. Moreover, the cocoon shrinkage caused severe mortality in *R. lezeyi*, suggesting that temporal and seasonal rainfall patterns may influence the persistence of *R. lezeyi* population via the alteration of flow regime and physicochemical environments in the stream. The present study also showed that the summer cocoon shrinkage of *R. lezeyi* may be caused by reduced osmotic gradient across the membrane during decreased flow and/or physical and physiological stresses due to high water temperature and low pH in that period. Further long-term studies are necessary to identify the detailed mechanisms of pupal cocoon shrinkage and its resultant effects on the *R. lezeyi* population dynamics.

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